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High affinity binding of α-bungarotoxin to the purified α-subunit and to its 27 000-dalton proteolytic peptide from *Torpedo marmorata* acetylcholine receptor. Requirement for sodium dodecyl sulfate

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Intact nicotinic acetylcholine receptor (AChR) tightly binds α-bungarotoxin. The two toxin-binding sites are presumed to be on the two α-subunits, either on or near the ACh-binding sites. Isolated α-subunits have been found to maintain weak binding to α-bungarotoxin (K_D ~0.2 μM). We describe here conditions under which the α-subunit and a 27 000-dalton proteolytic peptide bound α-bungarotoxin with high affinity. The four subunits of *Torpedo marmorata* AChR, as well as several proteolytic peptides of the α-subunit, were first purified by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. We found that the purified α-subunit (but not the β-, γ- or δ-subunits) and its 27 000-dalton peptide specifically bound ^{125}I-labeled α-bungarotoxin with K_D ~3 and 6 nM, i.e., about two orders of magnitude lower than the intact AChR. Nearly 100% of the sites were recovered. The recovery of this high affinity binding required the presence of SDS (~0.02%) but non-denaturing detergents had a strongly inhibitory effect. Unlabeled α-toxins competed with labeled α-bungarotoxin, α-bungarotoxin being more effective than all the other toxins tested. Decamethonium and hexamethonium competed efficiently with α-bungarotoxin binding but carbamylcholine had only a weak effect. The main immunogenic region of the AChR was only partially preserved since conformation-dependent monoclonal antibodies to this region bound the α subunit-toxin complexes, but much less efficiently than the intact AChR. We conclude that SDS can be advantageous to the recovery of high toxin binding to the α subunit which still has not completely recovered its native conformation.

Key words: α-bungarotoxin/α-subunit/acetylcholine receptor/protein conformation/SDS

Introduction

The nicotinic acetylcholine receptor (AChR) protein from the electric organ of *Torpedo* and *Electrophorus* is, thus far, the best characterized receptor for a neurotransmitter (reviewed in Karlin, 1980; Changeux, 1981; Conti-Tronconi and Raftery, 1982; Anholt *et al.*, 1983). Composed of four types of subunits in a molar ratio $\alpha_2\beta\gamma\delta$ (Reynolds and Karlin, 1978; Lindstrøm *et al.*, 1980; Saitoh *et al.*, 1980; Raftery *et al.*, 1980), the 250 000-dalton light form of the receptor can be reinserted into artificial lipid membranes (Wu and Raftery, 1979; Changeux *et al.*, 1979; Huganir *et al.*, 1979; Anholt *et al.*, 1980) and regulate cation fluxes in a manner similar to that found *in situ* in the subsynaptic membrane. In other words, this light form of the receptor protein is the smallest functional unit engaged in the physiological response.

The four types of subunits share considerable immunological (Tzartos and Lindstrøm, 1980) and amino acid sequence (Raftery *et al.*, 1980) similarities and thus may result from gene duplication. Each α-subunit carries at least part of one of the two acetylcholine-binding sites which regulate the opening of the ionophore (Changeux *et al.*, 1967; Karlin and Cowburn, 1973; Sobel *et al.*, 1977; Moore and Raftery, 1979). It can also be labeled by snake venom α-toxins which bind with high affinity (K_D ~1 nM to 20 pM) to the intact AChR (reviewed in Lee, 1979) and have proved invaluable tools for AChR research for more than a decade (Changeux *et al.*, 1970; Changeux, 1981). The same α subunits also contribute to the binding sites for non-competitive blockers of the ionic response (Oswald and Changeux, 1981).

To obtain information about the functionally important sites and about the pathogenicity of the molecule, Tzartos and Lindstrøm collected a large library of monoclonal antibodies directed against all four subunits (Tzartos and Lindstrøm, 1980; Tzartos *et al.*, 1981, 1982, and in preparation). Out of ~50 different monoclonal antibodies tested for their effect on the ion channel (Lindstrøm *et al.*, 1981; Tzartos and Lindstrøm, unpublished data), only two low affinity monoclonal antibodies, an anti-α and an anti-(α, β) were found. Both bound away from the ACh binding site, and both significantly inhibited the ionophore function.

A powerful method for identifying the particular role of each individual subunit of the AChR would be to achieve a reproducible reassembly of the AChR into a functional form from its dispersed subunits. Early reports (Meunier *et al.*, 1972) mentioned that the ability to bind snake venom toxins could be preserved after dispersion of *Electrophorus electricus* AChR into smaller mol. wt. units by sodium dodecyl sulfate (SDS). In a preliminary study, Huganir *et al.* (1981) reported that the β-subunit could be dissociated with urea and then reassociated to the remaining complex with a small recovery of toxin-binding activity. Haggerty and Froehner (1981) reported that α-subunit isolated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) exhibited some low α-bungarotoxin binding activity (K_D ~0.2 μM) after the elimination of SDS.

As an encouraging first step towards renaturing AChR from its SDS-dissociated subunits, we have found conditions under which the dissociated α-subunit binds α-bungarotoxin with high affinity (~3 nM). Unexpectedly, low concentrations of SDS appear essential for the recovery of this high affinity binding of α-bungarotoxin.

Results

Detergent requirements for the high affinity binding of α-bungarotoxin to the purified α-subunit

The α-subunit from *T. marmorata* AChR was purified by SDS-PAGE as described in Materials and methods and the binding of [^{125}I]α-bungarotoxin to the pure subunit assayed by filtration on DEAE filters which retain exclusively the bound toxin. Figure 1A shows that the 'denaturing' detergent SDS strikingly enhanced the binding of [^{125}I]α-bungarotoxin

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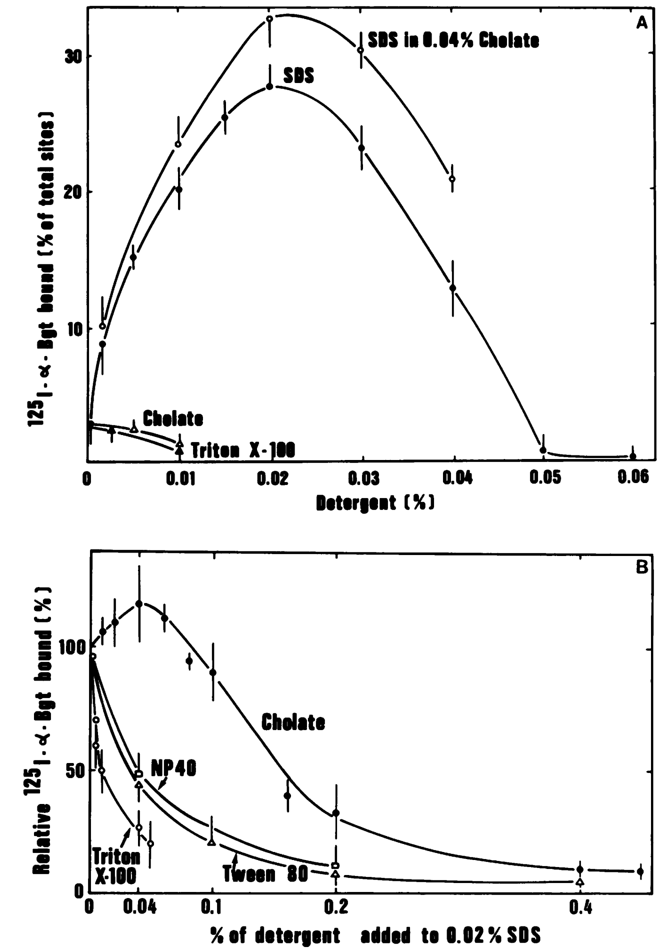

Fig. 1. Binding of [^{125}I]α-bungarotoxin to the purified α-subunit from *Torpedo* AChR in the presence of various concentrations of SDS, sodium cholate or Triton X-100. **(A)** Effect of single detergents. Purified α-subunit at final concentration 2 nM, or no subunit (for estimation of background) was mixed with [^{125}I]α-bungarotoxin (final concentration 2 nM) in 50 mM Tris buffer pH 7.4 supplemented with the indicated concentrations of SDS (●), cholate (△), Triton X-100 (▲) or 0.04% cholate plus the given concentrations of SDS (○). After 3 h incubation at ~24°C, the bound fraction of [^{125}I]α-bungarotoxin was measured by the DEAE-filter assay (see Materials and methods) and specific activity was calculated after subtraction of the background c.p.m. (1−3% of the total c.p.m.). The total initial binding sites were estimated from the α-subunit protein concentration (by Lowry) by assuming one binding site per 40 000 daltons. **(B)** Effect of non-denaturing detergents on the SDS-dependent [^{125}I]α-bungarotoxin binding to the α-subunit. Purified α-subunit (final concentration 2 nM) was mixed with [^{125}I]α-bungarotoxin (final concentration 2 nM) in 50 mM Tris buffer pH 7.4, 0.02% SDS supplemented with the indicated concentrations of cholate (●), NP-40 (□), Tween 80 (△), Triton (○). After 3 h incubation at ~24°C the specifically bound [^{125}I]α-bungarotoxin was measured by the DEAE-filter assay. ^{125}I-α-Bgt: [^{125}I]α-bungarotoxin.

up to an optimum concentration of 0.02%. Above this concentration the binding of the toxin declined to a negligible value. For the concentrations of α-subunit and toxin used in this experiment, only ~30% of the toxin-binding sites (estimated from protein concentration by the Lowry method) bound toxin at the optimum detergent concentration (Figure 1A). However, when an excess of toxin was used, >80% of the sites could bind [^{125}I]α-bungarotoxin.

When, instead of SDS, the 'non-denaturing' detergents Na^+ cholate or Triton X-100 were tested, no increase of toxin binding was observed. A small amount of α-bungarotoxin binding was detected in the absence of added SDS (9% of

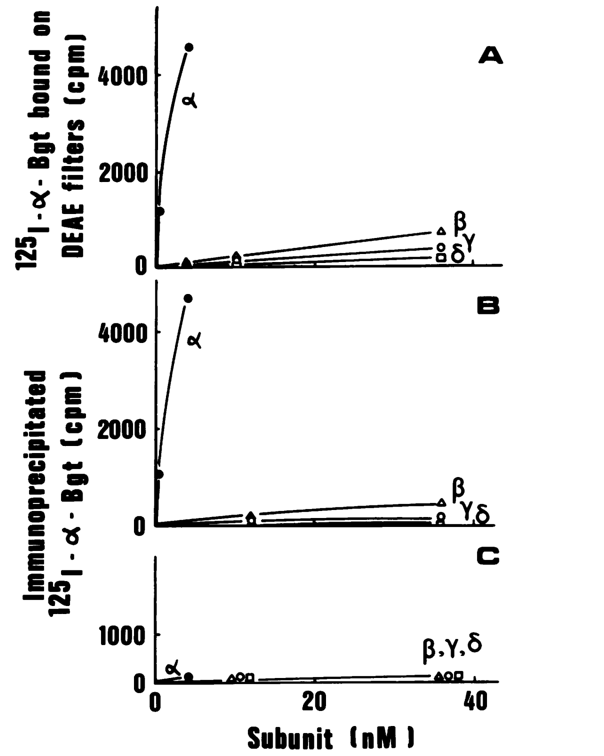

Fig. 2. Binding of [^{125}I]α-bungarotoxin to the four subunits of *Torpedo* AChR as measured by DEAE-filter assay and by immunoprecipitation using specific anti-subunit monoclonal antibodies. [^{125}I]α-bungarotoxin (final concentration 4 nM) was incubated with the shown final concentrations of α (●), β (△), γ (○) or δ (□) AChR subunits, or no subunit, in 30 μl samples, in 0.02% SDS−0.04% cholate−50 mM Tris buffer pH 7.4. After 3 h at ~24°C: **(A)** the specific toxin binding was estimated for some of the samples by the DEAE-filter assay; **(B)** to the remaining samples was added 5 μl of 1% dilution of anti-α-subunit monoclonal antibody 142 (Tzartos *et al.*, in preparation); or **(C)** 5 μl of 2% dilution of the anti-β monoclonal antibody 148, the anti-γ monoclonal antibody 132 or anti-γ, δ monoclonal antibody 7. After 2 h, normal rat serum and rabbit anti-rat γ-globulin were added in order to measure the immunoprecipitated subunit-toxin complex as described in Materials and methods. Only the anti-α-antibody could precipitate c.p.m. (shown in **B**); none of the other antibodies could (shown collectively in **C**). This suggests that the weak positive reaction of β-, γ- and δ-subunit preparations was due to contaminations by α-subunit.

maximal value in Figure 1A) but this fraction could have resulted from the presence of residual amounts of SDS in the stock solution of pure α-subunit.

Figure 1B shows the effect of a series of non-denaturing detergents (Na^+ cholate, Triton X-100, Nonidet P-40, Tween 80) on [^{125}I]α-bungarotoxin binding to the isolated α-subunit in the presence of the optimal concentration of SDS (0.02%). At low concentrations (up to 0.04%), Na^+ cholate slightly increased the binding but at higher concentrations, with the four detergents tested, a strong inhibition of α-toxin binding was observed. Accordingly, subsequent experiments were carried out in the presence of 50 mM Tris pH 7.4, 0.02% SDS and 0.04% Na^+ cholate.

Exclusive binding of [^{125}I]α-bungarotoxin to the α-subunit and not to the β-, γ- and δ-subunits of the AChR

The binding of [^{125}I]α-bungarotoxin to each of the four purified subunits was determined by filtration on DEAE papers (Figure 2A) and by precipitation with specific monoclonal antibodies directed against each subunit (Figure 2B, C). Toxin binds exclusively to the pure α-subunit since the low levels of toxin binding to the other subunit prepara-

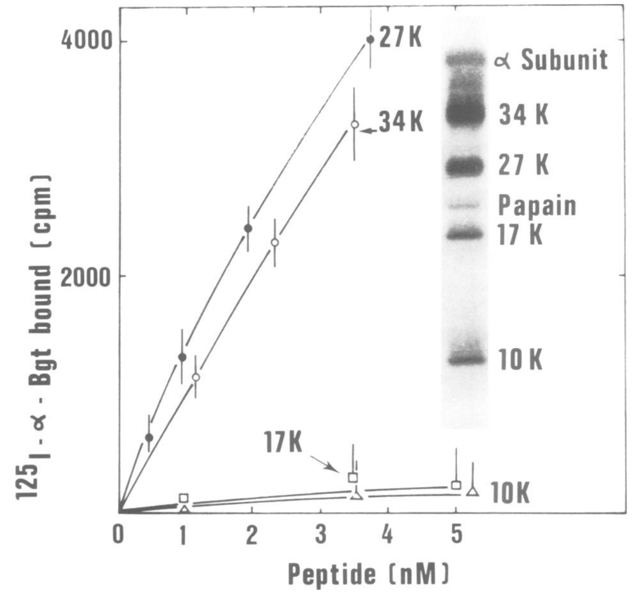

Fig. 3. [^{125}I]α-bungarotoxin binding to purified proteolytic peptides of the α-subunit. Purified α-subunit was papain treated and the peptides were isolated by preparative acrylamide gel electrophoresis (see Materials and methods). The shown concentrations of the 34-K (○), 27-K (●), 17-K (□) and 10-K (△) peptides in 0.02% SDS-0.04% Na^+ cholate were incubated with 4 nM [^{125}I]α-bungarotoxin for 3 h. The bound fraction of toxin was measured by the DEAE-filter assay (see Materials and methods).

tions revealed by the filter assay, were due to contaminating α-subunit as was shown by the monoclonal antibodies.

Binding of [^{125}I]α-bungarotoxin to proteolytic fragments of the α-subunit

As shown in Figure 3 (insert), treatment of the purified α-subunit by papain (as described in Materials and methods) yielded a discrete pattern of peptides of apparent mol. wts.: 34 000, 27 000, 17 000 and 10 000 daltons. This pattern did not change significantly over a range of papain concentrations of 10 – 200 μg/ml (not shown). Each of these peptides was purified by preparative polyacrylamide gel electrophoresis and tested for its ability to bind [^{125}I]α-bungarotoxin by the DEAE paper filtration assay under standard conditions. Figure 3 shows that, of the four peptides studied, only those of 34 000 and 27 000 daltons bound toxin.

Characteristic features of [^{125}I]α-bungarotoxin binding to the purified α-subunit and to its proteolytic fragments

Binding of [^{125}I]α-bungarotoxin to the purified α-subunit in the presence of SDS took place in the minute-hour time scale (Figure 4A). At 24°C, in the presence of 4×10^{-9} M [^{125}I]α-bungarotoxin, half maximal binding was reached in 10 min. Under these conditions the association rate constant was $k_1 = 0.95 \times 10^5$/M/s, ~30 times higher than the value found by Haggerty and Froehner (1981) for the SDS-free α-subunit solubilized in Na^+ cholate, but still 50 times lower than that determined by Wang and Schmidt (1980) for the intact AChR from *T. californica*. As expected, this rate decreased significantly at lower temperatures (Figure 4A).

Addition of a 100-fold excess of unlabeled α-bungarotoxin to the [^{125}I]α-bungarotoxin complex totally dissociated the complex with a half time for dissociation of 45 min (Figure 4B), three times longer than that for the Na^+ cholate solubilized α-subunit (Haggerty and Froehner, 1981). Under these conditions, the dissociation rate constant k_{-1} was

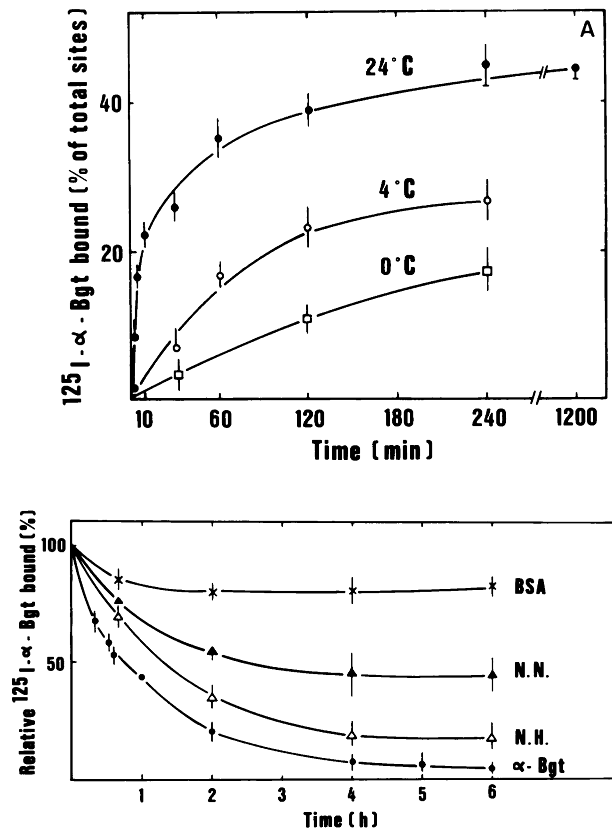

Fig. 4. Time course of [^{125}I]α-bungarotoxin binding to and dissociation from the α-subunit. **(A)** [^{125}I]α-bungarotoxin binding to the α-subunit at various temperatures. Purified α-subunit (final concentration 4.5 nM) or no subunit (for estimation of background) was incubated with [^{125}I]α-bungarotoxin (final concentration 4.5 mM) in 50 mM Tris buffer pH 7.4, 0.02% SDS – 0.04% cholate at 24°C (●), 4°C (○) or 0°C (□). After incubation for the periods shown, the specifically bound fraction of [^{125}I]α-bungarotoxin was measured by the DEAE-filter assay. **(B)** [^{125}I]α-bungarotoxin dissociation from the α-subunit by unlabeled toxins. Purified α-subunit (final concentration 3.6 nM) or no subunit (for estimation of background) was incubated with [^{125}I]α-bungarotoxin (final concentration 4 nM) in the same buffer as in A at ~24°C. After 4 h, excess unlabeled α-bungarotoxin (Boehringer, Mannheim) (final concentration 0.4 μM: ●), *N. haje* (8 μM: △), *N. nigricollis* (8 μM: ▲) α-toxins or BSA (8 μM: X) were added (time zero). At intervals the bound fraction of [^{125}I]α-bungarotoxin in 50 μl samples was estimated by the DEAE-filter assay.

found to be 2.6×10^{-4}/s.

The equilibrium dissociation constant K_D calculated from k_1 and k_{-1} was 2.7 nM, that is 10 – 130 times higher than the values reported with the intact AChR. Equilibrium binding of [^{125}I]α-bungarotoxin to the pure α-subunit and to the α-subunit in the mixture resulting from the dissociation of the AChR in SDS gave (four preparations) a similar value of 3 ± 0.9 nM (mean ± S.D.). The total concentration of active α-subunit estimated from the Scatchard plots was ~80 – 110% of the concentration estimated by the Lowry method in different experiments. Therefore, nearly all α-subunit molecules bound α-toxin with high affinity. Binding at equilibrium of α-bungarotoxin to the purified 27 000 proteolytic fragment of the α-subunit yielded a K_D of 6.1 ± 1 nM, close to that found with the pure subunit.

Toxins from various snakes were tested for their effect on the initial rate of binding of [^{125}I]α-bungarotoxin to the pure subunit. Unlabeled α-bungarotoxin was the most potent competitor among the snake toxins tested (Figure 5). The long α-toxin from *Naja haje* and the short α-toxin from *N. nigricol-*

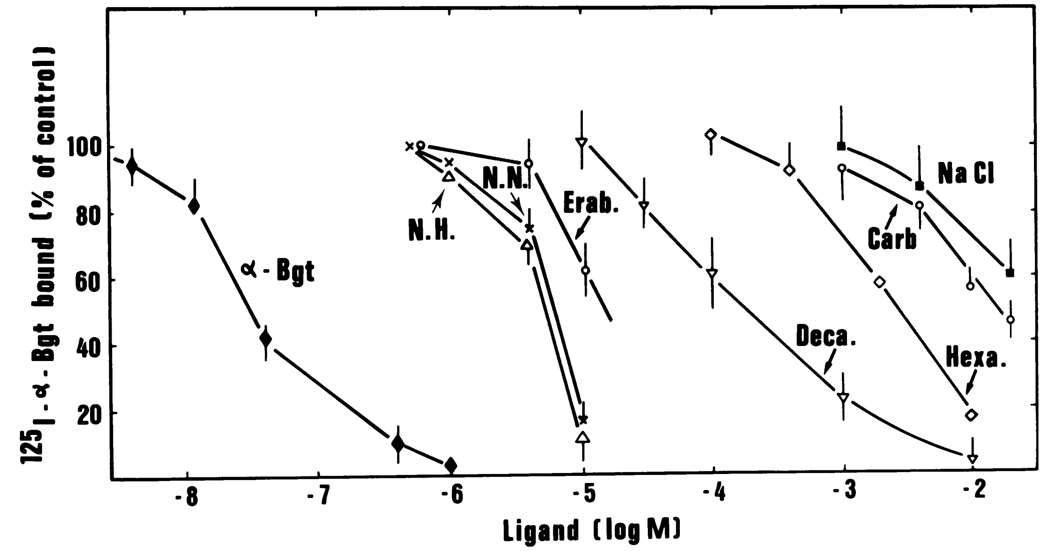

Fig. 5. Inhibition of [^{125}I]α-bungarotoxin binding to α-subunit by unlabeled ligands. Purified α-subunit (final concentration 4 mM) in 0.02% SDS – 0.04% Na^+ cholate was preincubated for 1 h with the indicated final concentrations of unlabeled ligands in total volume 50 μl. Then, 5 μl of 50 nM [^{125}I]α-bungarotoxin were added and incubated for another 10 min. The reaction was stopped with 1 ml 0.5% Triton 20 mM Tris buffer pH 7.4 and the bound fraction of [^{125}I]α-bungarotoxin was measured immediately by the DEAE-filter assay.

lis had a similar effect which was between the very potent long-chain α-bungarotoxin and the weak inhibitor short-chain toxin Erabutoxin b (Figure 5).

Among the several cholinergic agents investigated (Figure 5), the bis-quaternary ligands decamethonium and hexamethonium decreased the initial rates of [^{125}I]α-bungarotoxin binding to the pure α-subunit though to different extents. The effect of carbamylcholine was however hardly distinguishable from that of NaCl.

Binding of monoclonal antibodies to the pure α-subunit and to the 27 000-dalton peptide labeled with [^{125}I]α-bungarotoxin

The binding to the pure α-subunit and to the 27 000-dalton peptide of a large set of rat anti-AChR monoclonal antibodies was then compared with their binding to the AChR oligomer in 1% Na^+ cholate. All the antigens were labeled with [^{125}I]α-bungarotoxin. The results are presented in Table I.

The monoclonal antibodies raised by immunization of the rats with denatured AChR subunits efficiently reacted with the pure α-subunit in SDS (Table I, 1A). The 'renatured' pure subunit thus possesses structural features related to those of the denatured immunogen. With most of the antibodies a similar high reactivity for the 27 000-dalton peptide was observed. This is not surprising since this peptide contains 67% of the sequence of the whole subunit including part of its extracellular portion which carries the α-toxin binding site. However, several of these antibodies (8, 10, 61, 155, 157, 158, 164, 173) bound to a lesser extent to the 27 000-dalton peptide than to the pure α-subunit suggesting either a loss of antigenic determinants or a change of conformation (in the presence of SDS) caused by the proteolytic cleavage.

Table I (II.A) shows the binding of monoclonal antibodies directed against the main immunogenic region (MIR) of the Triton-extracted non-denatured AChR. The MIR is thought to be a conformation-dependent structure consisting of a group of neighbouring antigenic determinants (Tzartos and Lindstrøm, 1980; Tzartos *et al.*, 1981). Among the monoclonal antibodies specific for the MIR, several (2, 6, 47, 50) bound weakly but significantly to the 'renatured' α-subunit-toxin complex. This weak binding observed under the present experimental conditions appeared much stronger than that previously reported with the same antibodies but using denatured and reduced *T. californica* ^{125}I-labeled α-subunit under conventional conditions (0.1% SDS, 0.5% Triton X-100) (Tzartos and Lindstrøm, 1980; Gullick and Lindstrøm, 1982; Tzartos, unpublished data). Interestingly, the anti-MIR antibodies systematically did not bind, or bound to a much smaller extent to the 27 000 peptide than to the pure α-subunit, suggesting that the MIR was lost or disorganized as a consequence of the papain cleavage.

Among the monoclonal antibodies raised against the non-denatured AChR oligomer, three (5, 13, 19), which do not react with the MIR, bound strongly to the pure α-subunit in SDS. One of them behaved in an unexpected manner. Under our assay conditions, it appeared to bind more efficiently to the pure α-subunit in SDS than to the AChR oligomer. This particular antibody also reacted with the denatured α-subunit (Tzartos and Lindstrøm, 1980) but away from the regions involved in the MIR and the ACh-binding site. It is among the weakest antibodies to the AChR tested ($K_D \sim 4.5 \times 10^{-8}$ M) (Tzartos and Lindstrøm, 1981) but nevertheless one of the two which efficiently inhibit the $^{22}Na^+$-flux response of reconstituted AChR vesicles to cholinergic agonists (Lindstrøm *et al.*, 1981). Figure 6A shows that the difference in binding capacity of antibody 13 to the AChR oligomer and the pure α-subunit in fact results from the presence of Na^+ cholate in the assay medium. Na^+ cholate, used to keep the AChR oligomer in solution, strikingly inhibited the interaction of antibody 13 with the AChR oligomer (and also with the α-subunit in SDS as was found by using ^{125}I-labeled α-subunit; not shown). No effect of Na^+ cholate was found with any of the other antibodies tested.

Finally, some monoclonal antibodies directed against denatured β- and γ-subunits exhibited low but detectable binding to the α-subunit-toxin complex. Many monoclonal antibodies are known to cross-react with two or more

Table I. Binding of anti-AChR monoclonal antibodies to [^{125}I]α-bungarotoxin-labeled AChR, pure α-subunit and 27 000-peptide

Monoclonal antibodies	Binding to [^{125}I]α-bungarotoxin-labeled: AChR	α-subunit	27-K peptide
	(% of total complex ± S.D.)		
I. Immunogen: SDS-denatured subunits			
A. Antibodies to α-subunit			
8	62	43 ± 4	10 ± 3
10	55	33 ± 3	4 ± 2
61	61	61 ± 3	8 ± 2
142	99	96 ± 2	99 ± 3
147	100	74 ± 4	46 ± 5
149	85	59 ± 3	38 ± 2
152	86	52 ± 3	31 ± 2
153	97	66	43 ± 4
155	80	56 ± 2	23 ± 1
157	79	55 ± 2	21 ± 2
158	25	18 ± 2	2 ± 1
164	16	16 ± 1	1 ± 1
173	81	41	11 ± 4
187	100	82 ± 3	65 ± 3
B. Antibodies to β- and γ-subunits			
11 (anti-β)	41	1 ± 1	ND
123 (anti-β)	35	2 ± 1	ND
148 (anti-β)	89	5 ± 3	ND
168 (anti-γ)	66	0	ND
II. Immunogen: Triton-solubilized AChR			
A. Antibodies directed against MIR			
1	83	0	ND
2	82	3 ± 2	1 ± 1
4	89	0	ND
6	92	11 ± 4	1 ± 1
12	72	2 ± 1	ND
14	35	0	0
16	94	1 ± 1	0
17	73	2 ± 1	ND
35	63	2 ± 1	2 ± 1
47	91	15 ± 3	4 ± 3
50	86	8 ± 2	2 ± 2
B. Antibodies directed against other sites on MIR			
5	92	66 ± 3	45 ± 7
13	10	36 ± 2	22 ± 2
19	79	61 ± 2	36 ± 3

10–100 times excess of monoclonal antibody were incubated with 3 nM toxin-labeled antigen followed by immunoprecipitation with anti-antibody (see Materials and methods). AChR was in 1% cholate whereas α-subunit and 27-K peptide were in 0.015% SDS-0.04% Na$^+$ cholate. Binding to AChR was also tested in the presence of 0.01% SDS-0.04% Na$^+$ cholate which gave results similar to the above (not shown) except for monoclonal antibody 13 (see Figure 6).
ND, not determined.

subunits (Tzartos and Lindstrøm, 1980; Tzartos *et al.*, 1981, and in preparation; Gullick and Lindstrøm, 1982) because of amino acid sequence similarities among the four subunits (Raftery *et al.*, 1980).

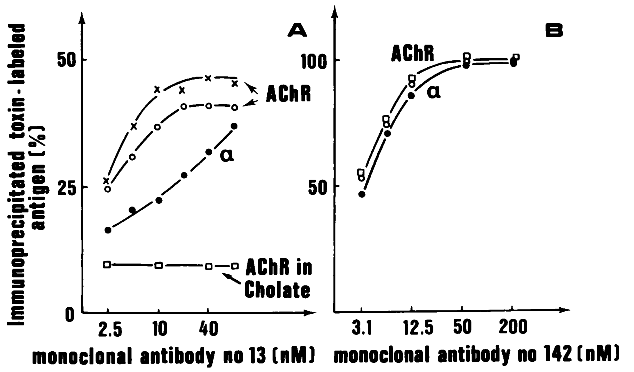

Fig. 6. Effect of detergents on binding of monoclonal antibodies 13 and 142 to AChR and α-subunit. The indicated concentrations of monoclonal antibody (**A**: 13; **B**: 142) were incubated with 4 nM toxin-labeled α-subunit in 0.015 SDS – 0.04% Na$^+$ cholate (●); or with 4 nM toxin-labeled AChR in 0.01% SDS – 0.04% Na$^+$ cholate (○), in 1% cholate (□) or in 0.5% Triton X-100 (X). Na$^+$ cholate has a strongly inhibitory effect on the binding of antibody 13 but not on 142 or on any other antibody tested (not shown). Binding of antibody 13 to ^{125}I-labeled α-subunit under the three conditions gave similar results (not shown).

Discussion

We describe here simple conditions that lead to the recovery of high affinity α-bungarotoxin binding to the isolated α-subunit and to its 27 000-dalton peptide in the presence of SDS. This detergent, widely used to denature proteins, actually maintains and promotes α-helical structures in some of them (Steele and Reynolds, 1979; Huang *et al.*, 1981) and has already served as the key-intermediate in refolding bacteriorhodopsin (Huang *et al.*, 1981). One possible explanation for the observed enhancement by SDS of the affinity of α-bungarotoxin for its site(s) is that it stabilizes α-helical structures in the isolated α-subunit necessary for α-bungarotoxin binding. However, it should be mentioned that the affinity of α-bungarotoxin for the pure α-subunit in SDS is still 1–2 orders of magnitude lower than that for the AChR oligomer and thus the site has not recovered all the properties characteristic of the native state of the receptor molecule. 'Non-denaturing' detergents, unlike SDS, inhibited toxin binding to the α-subunit.

The observation that a 27 000-dalton peptide resulting from papain cleavage of the α-subunit bound α-bungarotoxin with high affinity is also of interest. Gullick *et al.* (1981) have shown that a peptide of similar mol. wt. still carries maleimide benzyl-trimethylammonium (MBTA) after dissociation by SDS and papain treatment of MBTA-affinity labeled AChR. The two peptides are probably identical. They might also resemble the single large peptide (27 000 dalton) found by Bartfeld and Fuchs (1979) on SDS-polyacrylamide gels after extensive trypsin treatment of the AChR oligomer. This 27 000-dalton peptide thus carries at least part of the α-bungarotoxin binding site(s). Other groups or subsites with low affinity for toxin may exist on other parts of the α-subunit or on other subunits (Witzermann *et al.*, 1979; Karlin *et al.*, 1979; Oswald and Changeux, 1982). These sites might significantly contribute to the high affinity of the snake α-toxins for the native AChR, but might be too weak to be detected individually. Although all three loops of the α-toxins are considered to attach to particular sites on the AChR molecule (Tsetlin *et al.*, 1979), the long central loop appears the most critical (Lee, 1979) and indeed binds detectably as an isolated peptide (K_D

0.22 μM) to the intact AChR (Jillerat *et al.*, 1982). It would be interesting to know whether this central loop binds to the 27 000-dalton fragment of the α-subunit.

At variance with what is observed with the native oligomer (Weber and Changeux, 1974), the agonist carbamylcholine does not inhibit the initial rate of [125I]α-bungarotoxin binding to the α-subunit much more effectively than NaCl. However, the bis-quaternary agonist decamethonium and the antagonist hexamethonium effectively inhibited α-bungarotoxin binding. One explanation for this difference could be that on the isolated α-subunit in SDS, the anionic site where ACh or carbamylcholine binds does not coincide with the main area to which α-bungarotoxin attaches. In contrast, the second anionic subsite to which the bis-quaternary ligands decamethonium and hexamethonium bind could be more closely related to the α-bungarotoxin site. Of the four unlabeled α-toxins which were used to inhibit the initial rate of [125I]α-bungarotoxin binding (Figure 5) or to displace the already bound toxin (Figure 4B), α-bungarotoxin competed best, while the two short chain toxins were the least effective. Qualitatively, the situation resembles that found with the intact oligomer although the absolute dissociation constants found with the pure α-subunit in SDS are still several orders of magnitude higher. In summary, the isolated subunit in SDS has recovered several binding properties of the α-subunit in its native oligomeric arrangement but still differs from it.

The availability of a large library of monoclonal antibodies to conformation-dependent and independent sites on the various subunits of the AChR (Tzartos and Lindstrøm, 1980; Tzartos *et al.*, 1981, 1982, and in preparation) offered us the opportunity of investigating further the conformational state of the isolated α-subunit and of its peptides labeled by α-bungarotoxin. The monoclonal antibodies derived from rats immunized with denatured subunits bound well to the toxin-α-subunit complex (Table I). On the other hand, most conformation-dependent anti-α monoclonal antibodies bound weakly or undetectably. Nevertheless they gave a higher percentage of binding than the weak or undetectable binding observed with the 125I-labeled α-subunit from *T. californica* AChR (Tzartos and Lindstrøm, 1980; Gullick and Lindstrøm, 1982). It is interesting to compare the observation that the 'denaturing' SDS enhanced toxin binding to the α-subunit whereas the 'non-denaturing' detergents inhibited it, with the observation that Na^+ cholate dramatically inhibits binding of monoclonal antibody 13 to AChR (Figure 6) and to α-subunit. This monoclonal antibody is one of the only two which efficiently inhibit AChR function (Lindstrøm *et al.*, 1981). Binding of the other monoclonal antibodies was not inhibited by Na^+ cholate. Na^+ cholate is known to behave as a non-competitive blocker of the electroplaque response to cholinergic agonists (Brisson *et al.*, 1975; Heidmann *et al.*, 1980) and to stabilize the AChR in the absence of ligands in a low affinity 'degenerate' state (Heidmann *et al.*, 1980).

Several laboratories are currently studying *in vitro* and *in vivo* biosynthesis of the AChR subunits (Mendez *et al.*, 1980; Anderson and Blobel, 1981; Merlie *et al.*, 1982; Sumikawa *et al.*, 1981; Giraudat *et al.*, 1982) but they have not found conditions giving α-toxin binding to non-assembled α-subunit. The simple conditions we describe for high affinity toxin binding to the α-subunit and its peptide might eventually contribute to advancing these studies.

In conclusion, the present data: (i) show that low concentration of SDS can be advantageous for the recovery of some sites on the denatured AChR and might help as an intermediate step towards a complete renaturation of the AChR; (ii) provide a tool for studying α-bungarotoxin-AChR interactions.

Materials and methods

Preparation of AChR-rich membranes

AChR-rich membranes were prepared from *T. marmorata* electric organs according to Saitoh *et al.* (1980) and were further enriched in AChR by pH 11 treatment according to Neubig *et al.* (1979). The membranes were aliquoted and stored in liquid nitrogen.

Buffers. The buffer used was always 50 mM Tris pH 7.4 supplemented with detergents. The buffer for the final step of the filter assays and of the radioimmunoassays was always 0.5% Triton in 20 mM Tris buffer pH 7.4.

Preparation of AChR subunits

The above membranes (at final concentration of $\sim 1 \times 10^{-5}$ μM toxin-binding sites) were directly solubilized in 4% SDS (BDH chemicals), 50 mM Tris pH 7.4 buffer. After 10 min at 25°C, they were applied to a 10% acrylamide slab gel (Laemmli, 1970) without β-mercaptoethanol. After electrophoresis, parts of the gel were quickly stained with Coomassie blue and destained (20 min total) and used to localize the position of the subunits in the remaining large part of the unstained gel. The subunit-containing gel slices were broken into small pieces and shaken with 0.01% SDS for 1 h at room-temperature and 3–5 h at 4°C. The supernatants were then collected, frozen in aliquots and used without further treatment. The pieces of gel after elution had little if any remaining protein as judged by their failure to be subsequently stained. Protein concentration was determined according to the method of Lowry *et al.* (1951).

Preparation of α-subunit proteolytic peptides

The above α-subunit preparation (at ~0.05 mg/ml) was treated for 45 min at room temperature with 50 μg/ml papain (Sigma, 20.7 units/mg). The peptides were separated by electrophoresis on a 15% acrylamide slab gel. We used the same procedure to localize the position of the peptides except that because longer staining and destaining time periods were required, the unstained part of the gel was kept frozen to avoid diffusion of the peptide bands. The peptides were eluted from the minced gels as above by shaking with 0.01% SDS in 50 mM Tris for 4–6 h. Because the amounts of the eluted peptides were very low, protein concentrations were estimated indirectly from gel scans of stained gels.

Toxin-binding assay

For α-toxin binding measurements, the test toxin-binding preparation (AChR solubilized in Na^+ cholate, SDS-dissociated mixture of subunits, purified subunits or α-subunit protelytic peptides) was mixed with [125I]α-bungarotoxin (NEN, sp. act. 10–20 μCi/μg, repurified with a Sephadex G-50 column). After 3 h at 22–24°C, samples of 20–100 μl were transferred to Eppendorf tubes, their total radioactivity counted, then they were diluted with 1 ml 0.5% Triton X-100, 20 mM Tris pH 7.4, and immediately filtered through a triplet of Whatman DE-81 filters (2.3 cm diameter) prewashed with 3 ml of the same buffer. Non-specific (background) binding was considered to be the c.p.m. bound to filters from samples without AChR or subunit, but otherwise identical to the experimental samples. This non-specific binding (~2–3% of the total) was deducted.

Monoclonal antibodies and radioimmunoassays

The preparation and characterization of the monoclonal antibodies used in this paper has been described (Tzartos and Lindstrøm, 1980, 1981; Tzartos *et al.*, 1981, 1982, and in preparation). All monoclonal antibodies were derived from rats immunized with electric organ or muscle AChR or its subunits. Monoclonal antibody preparations used were obtained from media (containing fetal calf or horse serum) from cloned hybridoma cells. The Ig fractions were isolated and concentrated (~20 times) *via* 40–45% ammonium sulfate precipitation and dialysed against 100 mM NaCl, 10 mM NaN_3, 10 mM sodium phosphate buffer pH 7.5.

To test antibody binding, 20 μl of the [125I]α-bungarotoxin-subunit (or AChR or peptide) mixture, preincubated for 3 h, was mixed with 5 μl of a monoclonal antibody dilution (~1/1000–1/10 in 50 mM Tris, 3 mM NaN_3, pH 7.4 supplemented or not with bovine serum albumin (BSA) to a final protein concentration ~1 mg/ml) corresponding to a 10–100 times excess monoclonal antibody over the toxin-binding sites. For a few monoclonal antibodies limiting amounts of antibody were also tested. After 3 h incubation at room temperature, 5 μl of 1/50 dilution of normal rat serum and 8 μl of 1/2 dilution of rabbit anti-rat γ-globulin were added and further incubated for 45 min followed by dilution with 1 ml 0.5% Triton X-100, 20 mM Tris buffer pH 7.4, centrifugation and washing of the small pellets with 1 ml of the same buffer. Non-specific c.p.m. (which was deducted) was considered the c.p.m. contained in immunoprecipitates obtained with monoclonal antibodies 25 and 26 which are anti-*Electrophorus* AChR and do not bind to *Torpedo* AChR (Tzartos *et al.*, 1981).

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